Simple and rapid validated HPLC-fluorescence determination of perampanel in the plasma of patients with epilepsy

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A B S T R A C T

We present a simple and fast high-performance liquid chromatography method with fluorescence detection for the determination of the antiepileptic drug perampanel in human plasma. The chromatographic separation was performed on a Kinetex PFP (100 × 2.6 mm, 4.6 μm) column, using a mobile phase of sodium acetate 0.03 M pH 3.7 and acetonitrile (40/60, v/v), at a flow rate of 0.8 mL/min. Total chromatography time for each run was 5 min. Sample preparation (250 μL) involved only one simple precipitation step by acetonitrile spiked with mirtazapine as internal standard. The method was validated over a concentration range of 20–1000 ng/mL and successfully applied to measure perampanel concentrations in plasma samples obtained from patients with epilepsy. This assay combines the high specificity of fluorescence detection with a very simple and fast sample pretreatment and can offer real advantages over existing methods in terms of simplicity and transferability to a therapeutic drug monitoring setting.

1. Introduction

Perampanel (PER), [2-(2-oxo-1-phenyl-5-pyridin-2-yl-1,2-dihydropyridin-3-yl) benzonitrile hydrate] is a selective non-competitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist approved for the adjunctive treatment of patients with focal and generalized epilepsies aged ≥ 12 years [1]. It is extensively (> 90%) metabolized *via* cytochrome P450 CYP3A4/5 and concomitant intake of enzyme-inducing antiepileptic drugs (AEDs), such as carbamazepine (CBZ), phenytoin (PHT), oxcarbazepine (OXC) significantly reduces (up to 50–60%) plasma PER concentrations [2,3]. Preliminary post-marketing clinical experience shows a remarkable intersubject variability in drug response, both in terms of efficacy and tolerability [4,5], which may be partly ascribed to AED pharmacokinetic interactions. Therapeutic drug monitoring (TDM) has been suggested as an aid to optimizing individual patient's PER regimen [3,6].

The first published PER plasma assays, based on high performance liquid chromatography coupled with fluorescence detector (HPLC-F) [7,8], or tandem mass spectrometer (MS/MS) [8,9] required time-consuming liquid-liquid extractions (LLE). More recently, an HPLC-UV assay based on a precipitation step followed by solvent evaporation has been claimed to offer significant advantages in terms of simplicity of both sample pretreatment and laboratory equipment [10]. However, some methodological issues (an evaporation step of 1.5 h, the use of two columns in sequence to solve analyte resolution) may hamper its practical use.

We describe a simple, sensitive, highly specific method for PER determination in human plasma by HPLC-F coupled with a single deproteinization step that can offer real advantages over the existing methods in terms of simplicity and transferability to a TDM setting.

2. Materials and methods

2.1. Chemicals and materials

Perampanel was kindly provided by Eisai Co. Ltd (Kashima, Japan); the internal standard (IS) mirtazapine (MIR) was purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, ethanol, acetic acid and sodium acetate were purchased from Merck Millipore (Darmstadt, Germany). Ultrapure water was obtained from a MilliQ Gradient A10 apparatus (Merck Millipore). Frozen, drug-free plasma (blank plasma) for the preparation of calibrators and quality control (QC) samples was obtained from the blood bank of the Maggiore Hospital of Bologna, stored at −20 °C and thawed at room temperature before use.

2.2. Instrumentation and chromatographic conditions

A Perkin Elmer (Norwalk, CA, USA) 200 series HPLC system was used coupled with a series 200 fluorescence detector, set at excitation and emission wavelengths of 290 and 430 nm, respectively. The chromatographic separation was obtained by a Kinetex PFP 2.6 μm, 100 × 4.6 mm I.D. column (Phenomenex, Torrance, CA, USA) protected by a Security guard precolumn 4 × 3 mm (Phenomenex). The mobile phase was a mixture of sodium acetate buffer (30 mM, pH 3.7), filtered through a 0.22 μm membrane filter (GS type, Millipore) and acetonitrile (40:60, v/v). The flow rate was set at 0.8 mL/min.

2.3. Preparation of working solutions, calibration standards and quality control samples

Stock solution (1 mg/mL) and subsequent dilutions (10 μg/mL, 1 μg/mL working solutions) of PER were prepared by dissolving pure standards in ethanol. The internal standard stock solution (1 mg/mL) was prepared by dissolving 10 mg mirtazapine in acetonitrile. All solutions were prepared monthly and stored at 4 °C [7].

Plasma calibrators were prepared at final concentrations of 20, 40, 100, 200, 400, 1000 ng/mL by spiking 250 μL aliquots of blank pooled plasma with appropriate volumes of working solutions (10 μg/mL, 1 μg/mL). QC samples for method validation were similarly prepared to yield three drug concentrations of 20 (lower limit of quantitation, LLOQ), 100 (medium, MQC) and 1000 (high, HQC) ng/mL. A set of QC samples was also prepared at a concentration of 2000 ng/mL for the evaluation of dilution integrity. Calibrators and QC samples were freshly prepared for each batch.

2.4. Sample processing

Venous blood samples (5 mL) were drawn at 8 a.m. (around 8–10 h post ingestion of once-a-day bedtime PER dose) from ambulatory patients receiving chronic (> 1 month) PER therapy and attending their routine visit for TDM of other AED medication. Blood samples were transferred into heparinized tubes (8 IU heparin/mL blood) and centrifuged at 1500 × g for 10 min at 4 °C. A 500 μL plasma aliquot was separated, transferred into test tubes and stored at −20 °C for PER analysis. The procedure was approved by the Ethics Committee of the Bologna-Imola Local Health Trust (protocol number 16179). Written informed consent was obtained from each subject.

Plasma specimens (250 μL) were deproteinized by addition of 750 μL acetonitrile spiked with IS (10 μg/mL), vortexed for 30 s and then centrifuged at 1500 × g at 4 °C for 10 min. The supernatant was filtered (RC membrane filter 0.22 μm, Phenomenex) and 2 μL of the clean upper layer were injected into the chromatographic system.

2.5. Method validation

The method was validated for selectivity, linearity, dilution integrity, recovery, carry-over, stability [11], precision, accuracy and sensitivity [12]. The robustness of the method was checked against different operators [13].

3. Results and discussion

3.1. Chromatographic conditions

The described chromatographic conditions allowed an optimal separation of PER and IS, with mean ± SD ($n = 11$) retention times of 2.08 ± 0.02 min for PER and 4.72 ± 0.01 min for the IS (Fig. 1c).

3.2. Method validation

3.2.1. Selectivity
No endogenous interfering peaks were observed at the retention times of PER and IS from the analysis of 10 individual sources of
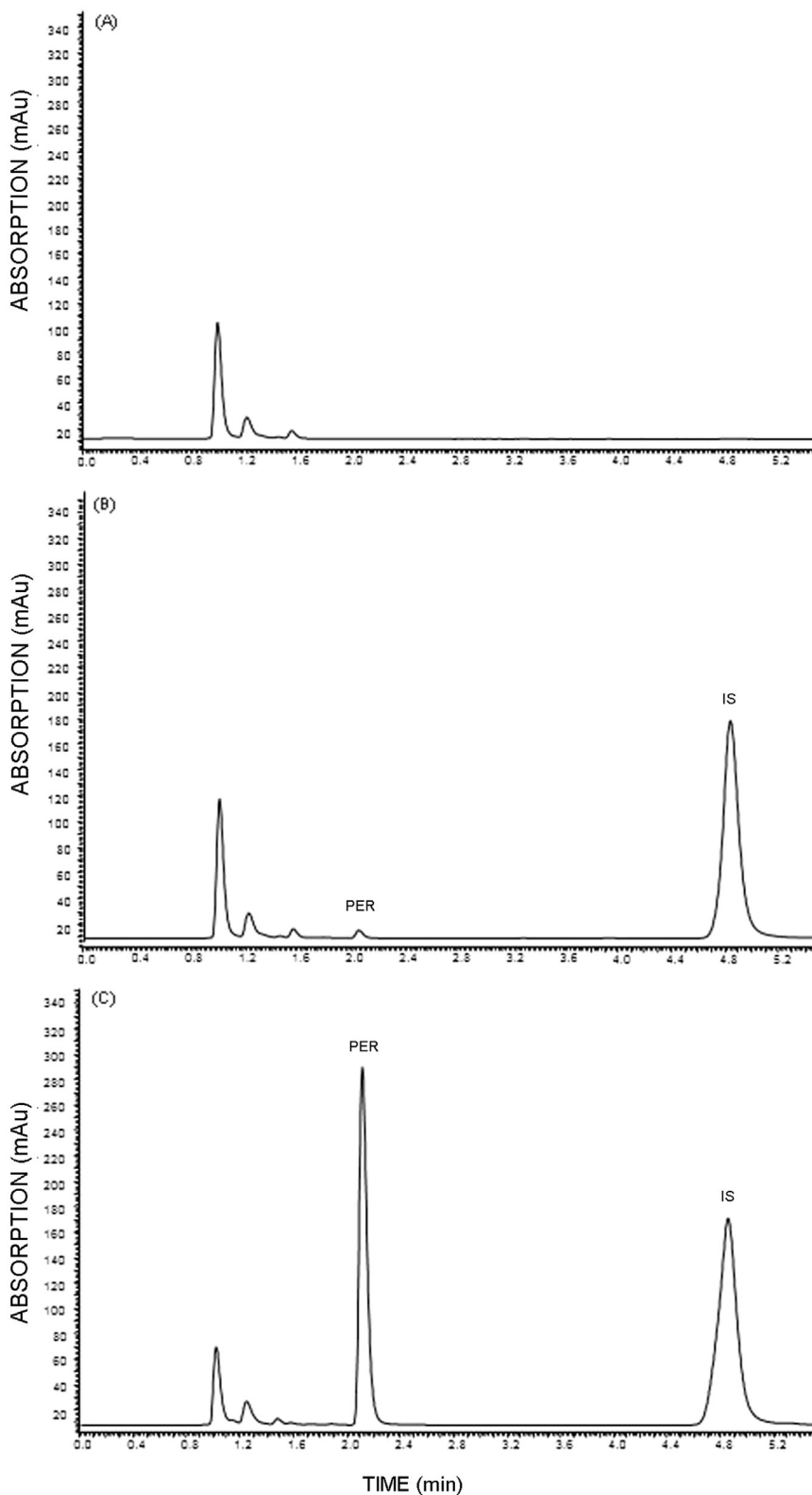
Fig. 1. Chromatograms of (a) blank plasma; (b) blank plasma spiked with perampanel at LLOQ (20 ng/mL) and internal standard; (c) plasma specimen of a patient treated with perampanel (6 mg/day), valproic acid (2500 mg/day), felbamate (2700 mg/day) and clobazam (30 mg/day): perampanel, 734.0 ng/mL. LLOQ, lower limit of quantitation.

blank plasma. A typical chromatogram is shown in Fig. 1a. Standard solutions of several commonly co-prescribed AEDs and their metabolites were injected to check for possible interferences. They included: CBZ (5 µg/mL); carbamazepine-10,11-epoxide (2 µg/mL); clobazam (500 ng/mL); ethosuximide (45 µg/mL); felbamate (40 µg/mL); gabapentin (2 µg/mL); 10-hydroxycarbazepine (10 µg/mL); lamotrigine (5 µg/mL); lacosamide (10 µg/mL); levetiracetam (15 µg/mL); norclobazam (1 µg/mL); OXC (1.5 µg/mL); phenobarbital (10 µg/mL); PHT (15 µg/mL); pregabalin (2 µg/mL); rufinamide (20 µg/mL); topiramate (3 µg/mL); valproic acid (50 µg/mL); zonisamide (15 µg/mL). None of the possibly co-prescribed drugs tested was detected over a 30-min run time.

3.2.2. Linearity, dilution integrity and sensitivity

Calibration curves showed a reproducible linear correlation over the range of 20–1000 ng/mL. Equations (mean ± SD, n = 6) of the regression lines were: $y = 0.0023 (\pm 0.0013) + 0.0011 (\pm 0.0001) x$, $r = 1.000 (\pm 0.0003)$. Accuracy and precision of dilution integrity determinations at the QC level of 2000 ng/mL (n = 5, dilution factor 1:1) were within the set criteria of ± 15% [11]. The lower limit of quantitation (LLOQ) was established at 20.0 ng/mL, defined as the lowest quantifiable plasma concentration of PER with an associated imprecision and inaccuracy ≤ 20%. Previous studies have reported lower LLOQ for PER plasma assays [7–9], however our LLOQ value is sufficient for the measurement of clinically relevant drug concentrations [3,10] in patients on chronic treatment. The lower limit of detection (LLOD) was set at 10 ng/mL, at a signal to noise ratio of 3:1.

3.2.3. Precision, accuracy, robustness and recovery

The results of precision and accuracy analyses for PER are reported in Table 1a. Both intra- and inter-assay imprecision (coefficient of variation, CV) and inaccuracy were ≤ 15% for the whole concentration range. Imprecision and inaccuracy of robustness analyses with respect to different operators were within ± 15% for all QCs as well (Table 1b).

Absolute recovery for PER was 99.5 ± 5.2% for LQC, 98.1 ± 1.4% for MQC and 101.3 ± 2.6% for HQC; recovery for the IS (10.0 µg/mL) was 80.5 ± 4.4%.

3.2.4. Carry over

Injection of blank samples (n = 6) after the highest calibrator did not show any signal > 20% of LLOQ at the retention time of PER, or greater than 5% at the retention time of IS (n = 6). The carry-over effect was therefore ruled out for both PER and the IS.

3.2.5. Stability

Perampanel plasma samples have been confirmed to be stable [7–10] under all tested conditions (2 h at room temperature, over two freeze-thaw cycles, after 30 days at −20 °C). Processed samples were stable after 24 h at the autosampler temperature of 4 °C (autosampler stability). Precision and accuracy of stability analyses were within ± 15% for both LQC and HQC.

3.2.6. Clinical application

The method was applied to determine PER plasma concentration in 37 plasma samples obtained from 30 patients (15 female, age 12–51 years, PER dose 2–10 mg/day) receiving different AED cotherapy. Steady-state PER plasma concentrations ranged from 28 to 1232 ng/mL, which are in line with previously reported data [3,6] at therapeutic dosages. The chromatogram obtained from one representative patient is shown in Fig. 1c. From preliminary analyses, median plasma concentrations of PER differed among patients grouped according to AED cotherapy (p = 0.01, one-way analysis of variance on ranks), otherwise comparable for age, sex, weight and PER daily dose. In agreement with the literature [3,4] lower values for median PER concentration were found in plasma samples from patients cotreated with enzyme inducing AEDs (CBZ, OXC, PHT, phenobarbital) (n = 18) compared with patients co-medicated with non-inducing AEDs, including lamotrigine, levetiracetam, lacosamide (n = 10): 175 ng/mL (25–75th percentiles: 115–358 ng/mL) *vs* 307 ng/mL (201–343 ng/mL). On the other hand, patients co-medicated with valproic acid (VPA), a broad-spectrum enzyme inhibitor [14], showed higher median plasma concentrations of PER (581 ng/mL, 241–819 ng/mL, n = 9), a finding not suggested from published data [3,4]. *In vitro* studies suggest that oxidative metabolism of PER is mainly mediated by CYP3A4/5, followed by glucoronide conjugation for some metabolites. However, the relative contributions of these metabolic pathways in humans remain unknown [15]. Our preliminary pharmacokinetic observations indicate the need for studies on a larger series of patients.

4. Conclusions

The proposed method combines the high specificity of fluorescence detection with a very simple and rapid sample pretreatment. Compared with previously reported HPLC-F [7] and HPLC-UV [10] assays for plasma PER, our procedure significantly simplifies and speeds up low volume plasma preparation, omitting time-consuming and expensive LLE and evaporation steps, which is helpful in a TDM setting. The method's quantitation range is adequate for PER purposes even in patients receiving low (2 mg) daily doses, as the LLOQ of the assay is well above the lowest concentration value of the putative "reference range" for PER of 180–980 ng/mL [3]. Finally, the method validation shows good intra- and inter-assay precision and accuracy, and good recovery, stability and robustness when tested with respect to different operators.

Acknowledgments

Table 1
Results of precision, accuracy and robustness analyses of perampanel assay.

a) Precision and accuracy of perampanel assay

Amount added to blank plasma (ng/mL)	Intraday (n = 6)			Interday (n = 18)		
	Calculated concentration (mean ± SD) (ng/mL)	Precision (CV%)	Accuracy (%)	Calculated concentration (mean ± SD) (ng/mL)	Precision (CV%)	Accuracy (%)
PER 20.0 (LLOQ)	19.55 ± 0.81	4.1	−2.3	18.39 ± 2.05	11.2	−8.1
100.0	98.00 ± 0.56	0.6	−2.0	96.18 ± 2.31	2.4	−3.8
1000.0	1009.53 ± 16.19	1.6	0.9	1020.05 ± 51.18	5.0	2.0

b) Robustness of perampanel assay with respect to different operators

Amount added to blank plasma (ng/mL)	(n = 9)		
	Calculated concentration (mean ± SD) (ng/mL)	Precision (CV%)	Accuracy (%)
PER 20.0 (LLOQ)	18.18 ± 0.76	4.2	−9.1
100.0	96.47 ± 3.12	3.2	−3.5
1000.0	981.77 ± 37.51	3.8	−1.8

Precision (CV%) = 100 × S.D./mean; Accuracy (%) = 100 × (mean concentration found – known concentration)/ known concentration); Interday (n = 18) = triplicate samples, over a series of six analyses on different days; Robustness (n = 9) = triplicate analyses for each low, medium and high QC sample; LLOQ = lower limit of quantitation; SD = standard deviation; CV = coefficient of variation.

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